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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

The major goal of the proposed research is to test the hypothesis that the estrogen-inducible transcription factor AREB6/ZEB contributes to the progression of breast carcinoma. In specific, I am investigating whether the expression of AREB6/ZEB becomes deregulated from estrogen control and, if so, at what stage of carcinogenesis. To test this, staged breast cancer samples are being collected and assayed by real-time PCR for the level of AREB6/ZEB mRNA. Furthermore, I am assessing relative estrogen levels by determining the amounts of pS2 and progesterone receptor mRNA in the biopsies, also by real-time PCR. The use of real-time PCR and the measurement of pS2 and progesterone receptor mRNAs represent changes in strategy from the original proposal. These changes were made because we were informed that serum estrogen levels were an unreliable measure of tissue estrogen levels (especially in postmenopausal women), because we were having difficulty collecting paired biopsy samples and serum, and because a real-time PCR machine became available. In addition, we are also measuring the amount of another family member ZEB-2, which has been implicated recently in invasion. At this point, I have virtually all the samples and reagents in hand to complete the proposed studies in the upcoming year.

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INTRODUCTION

Although estrogen is believed to contribute to the etiology of breast cancer, relatively little is known about the key genes that are regulated by the estrogen receptor. We have previously shown that estrogen induces the expression of the AREB6/ZEB (ZEB-1) transcription factor at the transcriptional level within two hours of treatment (1). These and other data led us to postulate that AREB6/ZEB plays a central role in transcriptional cascades triggered by estrogen. Our unpublished data also indicate that expression of AREB6/ZEB is deregulated, and high, in rapidly proliferating endometrial and ovarian cancers. Furthermore, microarray data suggest that AREB6/ZEB is more highly expressed in invasive/metastatic breast cancer lines compared to poorly invasive lines (2). In the systems where it has been tested, AREB6/ZEB is thought to act to promote cell proliferation and prevent differentiation (3-5). These observations led to our hypothesis that *AREB6/ZEB contributes to the progression and metastasis of breast cancer by promoting cell proliferation.* I propose that this is a consequence of AREB6/ZEB gene expression becoming independent of regulation by estrogen so that AREB6/ZEB is expressed at inappropriately high levels. Also, I anticipate that it is the deregulated expression of AREB6/ZEB that contributes to the estrogen-independent phenotype observed with some advanced breast cancers.

BODY

The specific research aims of this proposal are to I). *determine whether the level of expression of AREB6/ZEB correlates with the invasive and metastatic potential of breast cancers*, II). *investigate whether the AREB6/ZEB gene is amplified during breast cancer progression*, and III). *ascertain whether overexpression of AREB6/ZEB converts MCF-7 cells from a noninvasive to an invasive phenotype.* To complete these aims, four major Tasks were developed:

Tasks 1 and 2:

The first two tasks are designed to determine whether AREB6/ZEB gene expression becomes independent of estrogen in breast carcinomas and, if so, at which stage. The plan was to measure the amount of AREB6/ZEB mRNA and protein in staged human breast cancers and normal breast samples and to correlate that with serum estrogen levels. The accomplishment of these tasks has been somewhat delayed because of three changes in strategy as outlined below, but I now feel that I am on track to complete these goals within the upcoming year. Our original strategy was to collect serum samples from patients at the time of biopsy/surgery so that we could measure estrogen levels. This meant that collection of sufficient samples was delayed due to the necessity of coordinating both sample and serum collection from the patient. However, a few months ago Dr. Douglas Yee, an expert in breast cancer research in the Cancer Center here at the University of Minnesota, indicated that this was not the best approach to use because serum estrogen levels do not necessarily reflect tissue levels. He recommended measuring expression levels of estrogen-regulated genes such as pS2 or progesterone receptor (PR). Thus, I have changed my strategy and am now preparing to measure tissue levels of pS2 and PR mRNA and correlate their expression levels to that of AREB6/ZEB. Thus, we have stopped measuring

serum estrogen levels, which has had the added benefit of dramatically increasing our ability to collect staged breast cancer samples.

Another change in strategy also delayed the completion of the first two tasks. We had originally proposed to quantitate AREB6/ZEB mRNA using a quantitative competitive PCR assay. While a reasonable approach, a better one became available when a real time PCR machine was purchased by a neighboring laboratory. As real time PCR is much more quantitative and as four mRNAs can be analyzed in the same tube and at the same time, we decided to switch our method of analysis. This has necessitated designing primers for the four genes and establishing PCR conditions. The primers that are currently being tested are shown in Appendix 1. The conditions for measuring AREB6/ZEB mRNA using real-time PCR have been established and give a single, smooth curve as desired. As a preliminary test of their efficacy in measuring AREB6/ZEB mRNA in breast cancer, 4 samples have been assayed and the results are shown in Appendix 2. These data show that a single curve is obtained with the primers. However, the number of samples is too small to ascertain whether or not there is a correspondence between AREB6/ZEB mRNA levels and invasiveness of the cancers. Also, the control genes have not yet been assayed so we have no idea about the level of estrogen in these tissues. I am now in the process of collecting more staged breast cancer samples and extracting their RNAs so that they can all be assayed at the same time for the most consistent results.

The final change in strategy is relatively minor in practice but is of considerable intellectual importance. Recently, it has become increasingly apparent that the other family member, ZEB-2 (SIP), may play a role in oncogenesis (6). Therefore, it has become important to measure ZEB-2 expression as well as that of AREB6/ZEB (ZEB-1). Thus, I have designed real-time PCR primers for ZEB-2 in addition to those for AREB6/ZEB, pS2, and PR (Appendix 1) so that these mRNAs can be measured in the same reverse transcription reactions and at the same time. We feel that the information to be gleaned by comparing the relative expression of both family members is sufficiently important to justify this relatively minor change in strategy.

Task 3:

The third task is to determine whether the AREB6/ZEB gene is amplified in our breast cancer samples. This will be done by quantitative Southern blotting. This task is on hold until we collect and PCR assay the breast cancer samples. However, I have learned the Southern blotting procedure during the last year.

Task 4:

The goal of this task is to determine whether overexpression of AREB6/ZEB can make MCF-7 cells invasive. This requires a two-stage procedure to make a cell line in which the level of AREB6/ZEB is controlled by ecdysone treatment. In the first stage, a stable MCF-7 cell line is constructed containing the ecdysone receptor. The task of creating the ecdysone inducible expression vector is finished. I am now in the process of screening cells to identify those that have stably integrated the ecdysone receptor. The next step will be to insert the AREB6/ZEB expression vector into these cells. I anticipate that the lines will have been completed in the next few months and the actual experiments finished.

Task 5:

Preparation of the manuscript awaits collection of the data.

KEY RESEARCH ACCOMPLISHMENTS

- Development of an alternative strategy (measurement of the amount of the estrogen-regulated mRNAs for PR and pS2) for assessing estrogen levels in breast tissue. This method is more quantitative and enables us to collect breast cancers more readily.
- Learned real-time PCR
- Development of a real-time PCR assay for AREB6/ZEB. This method is more quantitative and enables us to measure the levels of other mRNAs at the same time.
- Design of real-time primers for ZEB-2, pS2, and PR. Establishment of real-time PCR reaction conditions are in progress.
- Collection of staged breast cancer samples and isolation of RNAs for testing is in progress.
- Learned the quantitative Southern blotting technique
- Creation of an ecdysone-inducible AREB6/ZEB expression vector
- Development of an MCF-7 cell line that is expressing the ecdysone receptor is nearly complete.

KEY TRAINING ACCOMPLISHMENTS

- Attended the Pathobiology of Cancer Workshop in Keystone Colorado and learned a great deal about cancer biology, met a number of researchers in the field, and began networking with students and faculty in the field of breast cancer research
- Attended weekly seminars in the University of Minnesota Cancer Center, especially those that pertained specifically to breast cancer.

REPORTABLE OUTCOMES

Abstracts:

Anose, B. M. and M. M. Sanders (2003). Steroid Hormones Regulate Expression of Human ZEB-1: Implications for Cancer Progression. 94th Annual Meeting of the AACR. Toronto, Canada , Rescheduled to Washington, DC (Abstract #5066).

Anose, B. M., M. Linnes, and M. M. Sanders (2003). Hormonal Regulation of ZEB-1 and Implications for Progression of Human Reproductive Carcinomas. IVth International Congress on Hormonal Carcinogenesis, Valencia, Spain (abstract accepted)

Invited Presentations:

03/28/03 "Steroid hormone regulation of human ZEB-1 and implications for cancer progression." Cancer Biology Research Club, University of Minnesota Cancer Center

Reagents:

- Ecdysone-inducible AREB6/ZEB expression vector
- Real-time PCR primers specific for AREB6/ZEB, ZEB-2, PR, and pS2

CONCLUSIONS

My initial progress in completing Tasks 1 and 2 in this proposal has been slower than anticipated due to the difficulties in obtaining enough staged breast carcinomas that had corresponding serum samples. However, since the change in strategy from measuring serum estrogen levels to measuring the amount of expression from estrogen-inducible genes in the cancers using real-time PCR, we have been able to collect many more samples and are in the process of preparing and analyzing them. I am also close to having the cell line constructed that is needed for Task 4. Thus, while this past year has largely been devoted to acquiring the reagents for the proposed experiments and learning the required techniques, I am now in the position of being able to collect data. Therefore, I anticipate that the project will be completed on schedule.

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APPENDICES

Appendix 1. Primers Designed for Real-Time PCR

Appendix 2. Example of Real-Time PCR Measuring AREB6/ZEB in Breast Carcinoma Samples.

Apendix 3. Abstract to be presented at the Annual Meeting of the American Association for Cancer Research

Anose, B. M. and M. M. Sanders (2003). Steroid Hormones Regulate Expression of Human ZEB-1: Implications for Cancer Progression. 94th Annual Meeting of the AACR. Toronto, Canada , Rescheduled to Washington, DC (Abstract #5066).

Appendix 4. Abstract to be presented at the IVth International Congress on Hormonal Carcinogenesis

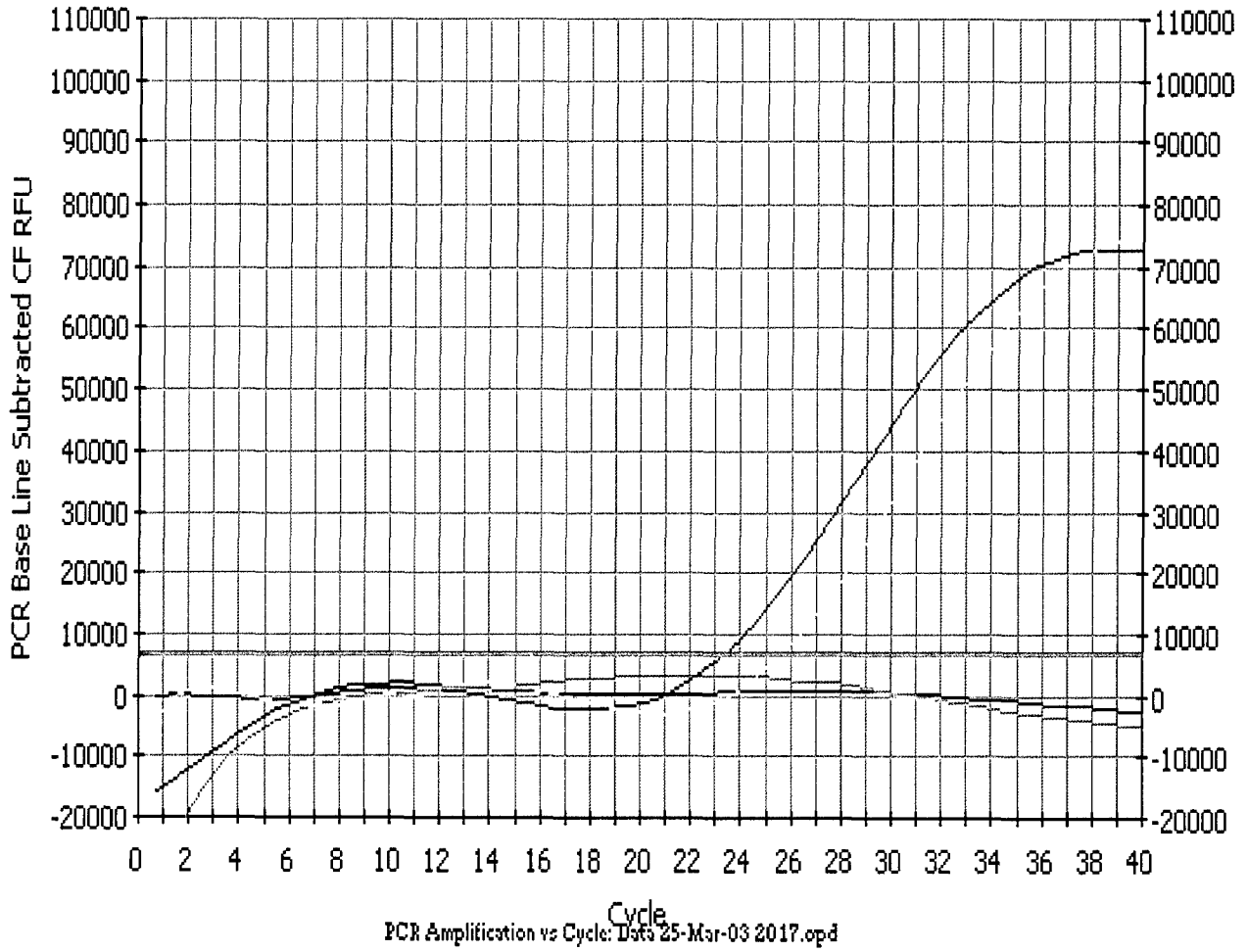
Anose, B. M., M. Linnes, and M. M. Sanders (2003). Hormonal Regulation of ZEB-1 and Implications for Progression of Human Reproductive Carcinomas. IVth International Congress on Hormonal Carcinogenesis, Valencia, Spain (abstract accepted)

Appendix 1. Real-Time PCR Primers

OLIGOMER NAME	**SEQUENCE**	T_M
ZEB-1 Forward	**5' TGC CCA GTT ACC CAC AAT CG 3'**	**58.6ºC**
ZEB-1 Reverse	**5' GGC TGA CCG TAG TTG AGT AGG 3'**	**57.8ºC**
ZEB-2 Forward	**5' CCA CGA GAA GAA TGA AGA GAA C 3'**	**54.4ºC**
ZEB-2 Reverse	**5' TGA GAT TAC CTG CTC CTT G 3'**	**55.8ºC**
pS2 Forward	**5' AGC AGA GAG GAG GCA ATG 3'**	**54.9ºC**
pS2 Reverse	**5' AAA CCA CAA TTC TGT CTT TCA C 3'**	**53.1ºC**
PR Forward	**5' TGG AGG CAG CAG TTC TAG 3'**	**54.5ºC**
PR Reverse	**5' CCA AAC AGG CAC CAA GAG 3'**	**54.2ºC**

All of the above PCR primers have been tested in real-time PCR reactions on reproductive carcinomas. Conditions for the ZEB-1 (AREB6/ZEB) primers have been determined such that a single curve is generated. Conditions for the other primers are still being established. The difficulty is in defining conditions that allow all 4 sets of primers to be assayed at one time.

Appendix 2. Real-Time PCR of Four Breast Cancer Samples



RNA was extracted from four breast cancer biopsy samples and subjected to real time PCR using the AREB6/ZEB (ZEB-1) primers. These are just test samples with incomplete pathology reports that I used to work out the assay conditions. The yellow line represents the results with a primary breast carcinoma and the three others are from metastatic carcinomas. The purple and yellow lines are exactly the type of profile that one would expect if the primers are performing as desired. The other two lines are the results one gets when there is no detectable specific RNA (AREB6/ZEB) in the sample. Controls (pS2, progesterone receptor and ZEB-2) have not been run on these samples yet.

Appendix 3. Abstract to be Presented at the 94th Annual Meeting of the American Association for Cancer Research

Steroid Hormones Regulate Expression of Human ZEB-1: Implications for Cancer Progression.

Anose, B.M. and M. M. Sanders, Department of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota, Minneapolis, MN 55455

The human Zinc finger E-box Binding (ZEB)-1 transcription factor belongs to an ever-growing family of zinc-finger homeodomain proteins that are involved in critical developmental processes. From flies to humans, these factors have been shown to regulate a number of important pathways in processes as diverse as myogenesis, hematopoeisis, skeletal patterning, and gonadal development. Work in our lab has shown that ZEB-1 is often deleted or deregulated in various female reproductive carcinomas. It has also been reported that expression of ZEB-1 is significantly increased in invasive breast cancers, and even more so in invasive, metastatic tumors. In addition to this, the ZEB-1 gene has been chromosomally mapped to a locus deleted in some prostate cancers. Despite these findings, very little is known of the mechanisms by which ZEB-1 is regulated or the downstream targets of this important transcription factor.

A number of experiments were undertaken by our lab to elucidate the mechanisms of human ZEB-1 gene regulation and to study its role in reproductive carcinomas. We have previously reported that the chick homolog of ZEB-1 is induced by estrogen in primary chick oviduct cells. To investigate whether the human gene is also regulated by estrogen, the human Ov266 ovarian carcinoma cell line was treated with estrogen over a time course spanning 0-24 hours. Reverse Transcription (RT)-PCR analysis revealed the endogenous ZEB-1 gene is induced by estrogen within 3 hours of treatment. To investigate whether the human gene is regulated by any other class of steroid hormones, a dose response assay was performed to study its possible regulation by androgen. RT-PCR analysis revealed the ZEB-1 gene is also induced by 5 nM dihydrotestosterone (DHT) in the human PC-3/AR prostate carcinoma cell line.

As the human ZEB-1 promoter had never been identified, experiments were undertaken to clone this and the upstream regulatory region. Approximately 1000 base pairs of the region upstream of the translation start site of human ZEB-1 were successfully cloned. Analysis of this region reveals a number of putative estrogen, progesterone, and androgen response elements. Experiments are underway to test the functionality of these elements. Concurrently, the transcription start site is being determined. Experiments are also being performed to further investigate the presence and expression of ZEB-1 in various stages of human reproductive carcinomas. Preliminary results indicate that the ZEB-1 gene is present in rapidly proliferating female reproductive carcinomas and deleted in slow-growing cancers. Current experiments involve measuring the levels of ZEB-1 mRNA in reproductive cancers at each stage of progression.

Our results show that the ZEB-1 gene is regulated by at least two classes of steroid hormones. Furthermore, we hypothesize that the loss of regulation by steroid hormones contributes to the progression of reproductive carcinomas due to inappropriate ZEB-1 expression.

Appendix 4. Abstract to be presented at the IVth International Congress on Hormonal Carcinogenesis

HORMONAL REGULATION OF ZEB-1 AND IMPLICATION FOR PROGRESSION OF HUMAN REPRODUCTIVE CANCERS

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The human Zinc finger E-box Binding (ZEB)-1 protein belongs to a family of transcription factors involved in critical developmental processes. Yet little is known of the mechanisms by which ZEB-1 is regulated. Our lab has recently demonstrated the expression of ZEB-1 is induced by estrogen in the ovarian cancer cell line Ov266, and it is regulated by dihydrotestosterone (DHT) in the human PC-3/AR prostate carcinoma cell line. Interestingly, a dose-response assay indicates the expression of ZEB-1 is induced by 5 nM DHT and repressed at higher dosages. Cloning and analysis of approximately 1000 bp upstream of the translation start site of hZEB-1 revealed a number of putative estrogen and androgen response elements. Transient transfection assays indicate that this region is sufficient to confer responsiveness to both steroids.

To determine whether expression of ZEB-1 could serve as a marker of tumor progression, real-time PCR assays were performed on various stages of human reproductive carcinomas. Preliminary results indicate that expression of ZEB-1 increases as the normal ovary transforms to a primary carcinoma and continues to increase as the cancer progresses to an invasive and finally a metastatic state. There is an approximate 12-fold elevation in the expression of ZEB-1 in metastatic ovarian carcinoma relative to its expression in *in situ* cancers. This technique is currently being utilized to investigate the potential changes in ZEB-1 expression in breast and prostate cancer during the progression of these diseases. These data raise the possibility that overexpression of ZEB-1 contributes to the progression of reproductive carcinomas.